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Comparative Study of DNA Preservation under Various Conditions on Local Egyptian Cowpea Germplasm

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Abstract

This study were to study the impact of storage conditions on DNA quality in different materials, the integrity and quality of stored DNA; as well as determining the best way to store of DNA extracts in some Cowpea plants collected from different sites in Egypt. Ten germplasm representing *Vigna radiata* and *V. unguiculata*, procured from local regions by National Gene Bank (NGB), Agricultural Research Centre (ARC), Giza, Egypt. The DNA from each treatment was stored in different buffers for at least a year at LN, -20°C, -80°C and room temperature (RT) without special control of temperature or humidity. In order to simulate and evaluate the long-term storage samples subjected to high temperature over a period of time by extrapolation could correspond to 100 years at 25°C. These experiments were conducted on the samples of ten samples of *Vigna* at 65°C for one day in hot-plate. The obtained results, the protectant trehalose were the highest value of DNA concentration (43.6±1.1 ng/μL) at room temperature, following by PVA scored 43.5±1.0 ng/μL at RT and TE buffer showed 43.5±0.7 ng/μL at -20°C, for stored DNA. The average mean of PVA and trehalose were 43.5±1.1 ng/μL more than TE-buffer. For stored tissue, the high mean (43.5±0.8 ng/μL) value of DNA concentration scored with the silica-gel and herbarium specimens, respectively. DNA samples of ten samples under study were also subjected to PCR analysis using SSR to determine whether the DNA was degraded. While no degradation was observed for DNA stored under different conditions, special in dry conditions (≤ 5% humidity). To assess its effects on DNA stability during storage at different conditions under controlled humidity. It was very interesting to note that DNA samples stored in the presence or absence of protectant additives exhibited no detectable degradation, whereas in the absent of, DNA was degraded and appeared as a smear on an agarose gel.

Keywords: Cowpea, SSR, DNA Preservation, DNA Storage.

Introduction:

Biodiversity is very important for conserving genetic diversity present in and available to our current and potential crop species. Cultivated crops are extremely inbred for factors like yield, uniform flowering and height, and cosmetic features of products. This narrow genetic base has resulted in several disastrous crop failures (Adams, 1997). It is well known that there are two approaches to conservation of PGR - ex situ and in situ. Ex situ conservation approach generally comprises the following methods: seed storage, field gene-bank, in vitro storage, pollen storage, DNA storage and botanical gardens.

The genomic DNA extraction is a primordial stage in several practices and genetic analyses such as those that involves the use of molecular markers, which has had a growing application in the plant systematics and in the population analysis. Studies based in molecular markers can be used successfully in phylogenetic analyses, serving as a great help in matters unresolved by traditional

methods of analysis (Salamin et al., 2005; Chapman et al., 2007); the characterization of genetic diversity of germplasm banks, discrimination among germplasm, detecting duplications, mixed seeds, drift and uncontrolled intersections (Efombagn et al., 2008 and Xie et al., 2010); in determining the degree of relatedness between individuals; the study of population's genetic structure, genetic effects of fragmentation and gene flow (Takayama et al., 2008; Bittencourt and Sebbenn, 2009); construction of genetic maps (Song et al., 2004; Moretzsohn et al., 2005); in studies of polyploidy (Doyle et al., 2002 and Joly and Bruneau, 2004) and speciation (Barraclough and Vogler, 2000). All molecular analyses, to be successful, depend on obtaining DNA samples of quality. Therefore, procedures are needed for efficient collection and storage of plant material, as well as isolation of the DNA molecule (Witono and Kondo, 2006; Whitlock et al., 2008). Experience shows the use of fresh material is ideal for holding the DNA isola-

tion (Sytsma et al., 1993). However, in studies that involve the collection of wild plants it is not always possible, since in most cases the populations are far from the research laboratory. An alternative is to perform the freezing of plant material in liquid nitrogen during collection in the field. However, this practice is not feasible in many cases, given the difficulty and danger of transporting liquid nitrogen container in rough terrain and difficult access. Thus, a method much used in the preservation of plant tissue for subsequent DNA extraction is rapid dehydration in silica gel (Witono and Kondo, 2006; Whitlock et al., 2008). This technique is generally simple and efficient, since the dehydrated state, the DNA is less susceptible to chemical or enzymatic degradation (Murray and Thompson, 1980). However, some species do not respond well to this type of conservation, with losses in quality of the obtained DNA. Other ways of preserving material may be used; however, not all are simple or require the availability of some specific equipment such as freeze dryers.

ment such as freeze dryers.

The progress in genetic engineering has resulted in breaking down the species and genus barriers for transferring genes (National Research Council, 1993). Transgenic plants have been produced with genes transferred from viruses, bacteria, fungi and even mice. Such efforts have led to the establishment of DNA libraries, which store total genomic information of germplasm (Mattick et al., 1992). However, strategies and procedures have to be developed on how to use the material stored in the form of DNA. Therefore, the role and value of this method for PGR conservation are not completely clear yet (Adams et al., 1992).

This study therefore were to study the impact of storage conditions on DNA quality in different materials, the integrity and quality of stored DNA; as well as determining the best way to store of DNA extracts in some Cowpea plants collected from different sites in Egypt.

Materials and methods:

Ten germplasm representing *Vigna radiata* and *V. unguiculata* subspecies cv-group: *unguiculata*, procured from local regions by National Gene Bank (NGB), Agricultural Research Centre (ARC), Giza, Egypt. The studied accessions of genus *Vigna*, collected date and area were presented in Table (1).

Table (1): List of studied accessions of genus *Vigna*.

ID	Taxa	Accession No.	Collected date	Collected area
1	<i>Vigna radiata</i> Varity: <i>radiata</i>	19538	2005	Giza
2		19537	2004	Giza
3	<i>Vigna unguiculata</i> Subspecies: <i>unguiculata</i> Cv-group: <i>unguiculata</i>	14176	2008	Dakhla
4		13884	2008	Dakahlvia
5		101	2010	Luxur
6		27301	2007	Sohag
7		26983	2007	Qena
8		26981	2007	Qena
9		26945	2007	Aswan
10		26738	2006	Menvia

DNA Extraction and storage:

Total genomic DNA from 5 g of leaf tissue per germplasm was extracted following the Zymo Research. Electrophoresis was made on 1% agarose gel electrophoresis at 100 Volt for 30 min. The total genomic DNA was diluted to 10 ng/μl for PCR analysis.

The herbarium voucher-specimens are deposited at the herbarium Department of Taxonomy, National Gene Bank (NGB. While, extracted gDNA was used to test different treatments and storage conditions. The used protective-agents applied to add with DNA samples trehalose (5μL of 10%), according to Taylor et al., 1994; and McGinnis et al., 2005; Tris-EDTA (TE) buffer; Polyvinyl Alcohol (PVA; 5μL of 1%), according to Papancea et al., 2008. Samples were stored in -20°C or -80°C and liquid nitrogen (-196°C).

Measuring the concentration of DNA samples

The herbarium voucher-specimens are deposited at the herbarium Department of Taxonomy, National Gene Bank (NGB. While,

extracted gDNA was used to test different treatments and storage conditions.

Simple Sequence Repeats (SSR-PCR) analysis:

Ten microsatellite primers (Table 2) were evaluated to amplify the success PCR after the storage. The PCR reactions were performed in a 20 μl reaction mixture containing 10 ng template DNA, 200 μM dNTPs, 250 nM of each primer, 1.5 mM MgCl₂, 1x PCR buffer and 1 unit Taq DNA polymerase. The PCR amplification was performed with an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 53 to 60°C (depending on the primer) for 1 min, 72°C for 2 min, and a final extension at 72°C for 5 min before cooling at 4°C.

Statistical analysis:

Basing on the averages of all attributes, data set were fed into SPSS (version. 14.0) and StatistiXL adding in Microsoft Excel (Kovach Computing Service 2013, version. 1.8) Program.

Table 2. Primer sequence, annealing temperature and number of alleles of *Vigna* specific SSR markers.

Primer		Primer sequence	Tm
Eh01	Forward:	TCTTGTCATTTAGCACTTAGCACG	60
	Reverse:	TTGTTGTTTACTAAGAGCCCGTGT	
Eh02	Forward:	GAGTTTACAACAGATGGGGCTAA	60
	Reverse:	AGGTCTTGATTGACTTTCTGGGT	
Eh03	Forward:	GGTTAGCATCTGAGCTTCTTCGTC	60
	Reverse:	AACACCCGCCTCTTTCTCC	
Eh04	Forward:	TCAGCAACCTTGCATTGCAG	57
	Reverse:	TTCCCGTCACTCTTCTAGG	
Eh05	Forward:	TTCCATCTATAGGGGAAGGGAG	60
	Reverse:	GCTATGATGGAAGAGGGCATGG	
Eh06	Forward:	AATTGCTCTCGAACCAGCTC	57
	Reverse:	GGTGTACAAGTGTGTGCAAG	
Eh07	Forward:	GCTTGCATCACCCATGATTC	57
	Reverse:	AAGTGATACGGTCTGGTTCC	
Eh08	Forward:	CGTTCGAGTTTCTTCGATCG	57
	Reverse:	ACCATCCATCCATTTCGCATC	
Eh09	Forward:	TGGGCTACCAACTTTTCCTC	57
	Reverse:	TGAGCGACATCTTCAACACG	
E013	Forward:	TCGCAAAGTTGCCAGTCAGT	42
	Reverse:	TAGAAGGAAGGAGGGCATG	

Results and Discussion:

The results can be represented as an interaction of several independent factors affecting DNA preservation, such as concentration, protective agent, and temperature, represented onto Fig. 1. The DNA from each treatment was stored in different buffers for at least a year at LN, -20°C, -80°C and room temperature (RT) without special control of temperature or humidity. In order to investigate whether the storage time affected sample recovery and

rehydrated samples of DNA were analyzed using specific microsatellite primers, see Table 2.

In order to simulate and evaluate the long-term storage samples subjected to high temperature over a period of time by extrapolation could correspond to 100 years at 25°C. These experiments were conducted on the samples of ten germplasm of *Vigna* at 65°C for one day in hot-plate.

Figure (1): Ideogramical representation of DNA concentration.

The fluorometric analysis indicated that the overall there was little or no difference in the amount of DNA recovered as compared to the control samples. The obtained results are shown in Table 3. The obtained results, the protectant trehalose were the highest value of DNA concentration (43.6 ± 1.1 ng/μL) at room temperature, following by PVA scored 43.5 ± 1.0 ng/μL at RT and TE buffer showed 43.5 ± 0.7 ng/μL at -20°C , for stored DNA. On the other hand, the average mean of PVA and trehalose were 43.5 ± 1.1 ng/μL more than TE-buffer. For stored tissue, the high mean (43.5 ± 0.8

ng/μL) value of DNA concentration scored with the silica-gel and herbarium specimens, respectively. In general, the control appeared the highest value of DNA concentration (43.7 ± 1.1 ng/μL) comparing with the whole treatments (Fig. 1); following by the value of trehalose (43.6 ± 1.1 ng/μL).

After a month of storage at a different degree of temperatures, no degradation was seen whereas, as shown in Fig. (1), no visible differences of the samples occurred after when compared to the control sample.

Table (3): DNA concentration (ng/μL) of samples.

Accession		1	2	3	4	5	6	7	8	9	10	Mean	SE
Control	4	47.0	42.4	51.3	49.2	42.7	35.4	38.6	43.5	45.2	41.6	43.7	1.1
PVA	-20	46.8	42.0	51.2	48.9	42.3	35.0	38.4	43.2	45.1	41.3	43.4	1.1
	-80	46.7	42.1	50.4	49.0	42.5	35.1	38.4	43.1	45.0	41.2	43.4	1.0
	RT	46.2	42.2	51.0	48.8	42.6	35.3	38.5	43.3	45.1	41.6	43.5	1.0
	Mean	46.6	42.1	50.9	48.9	42.5	35.1	38.4	43.2	45.1	41.4	43.5	1.1
	SE	0.2	0.1	0.2	0.1	0.1	0.1	0.0	0.1	0.0	0.1		
Trehalose	-20	46.9	42.2	51.1	48.6	42.2	35.2	38.2	43.3	45.1	41.5	43.4	0.7
	-80	46.9	42.3	51.0	48.7	42.1	35.1	38.1	43.2	44.8	41.0	43.3	1.1
	RT	47.0	42.4	51.2	49.0	42.6	35.4	38.5	43.4	45.1	41.5	43.6	1.1
	Mean	46.9	42.3	51.1	48.8	42.3	35.2	38.3	43.3	45.0	41.3	43.5	1.1
	SE	0.0	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.2		
TE-buffer	-20	46.5	42.3	51.1	48.7	42.6	35.1	38.5	43.3	45.1	41.4	43.5	0.7
	-80	46.2	42.2	51.2	48.9	42.5	35.0	38.4	43.2	45.0	41.4	43.1	1.1
	RT	46.0	41.6	50.0	48.2	42.0	34.7	38.2	43.0	44.4	40.0	42.8	1.1
	Mean	46.3	42.0	50.8	48.6	42.4	34.9	38.4	43.2	44.8	40.9	43.2	1.0
	SE	0.3	0.2	0.4	0.2	0.2	0.1	0.1	0.1	0.2	0.5		
LN	-20	45.7	41.8	51.0	49.0	42.6	35.2	38.4	43.4	45.0	41.3	43.3	0.7
	-80	45.8	41.8	51.1	48.7	42.5	35.0	38.6	43.2	44.9	41.2	43.3	1.0
	Mean	45.8	41.8	51.1	48.9	42.6	35.1	38.5	43.3	45.0	41.3	43.3	1.0
	SE	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0		
Silica Gel	RT	46.7	42.1	51.1	49.1	42.5	35.2	38.4	43.4	45.1	41.2	43.5	0.8
Specimens	RT	46.7	42.1	51.1	49.1	42.5	35.2	38.4	43.4	45.1	41.2	43.5	1.1

SE=Standard Error; PVA=Polyvinyl Alcohol; TE-buffer= Tris-EDTA buffer; LN=Liquid Nitrogen.

As any cut in the target sequence will prevent its amplification, DNA samples of ten accessions under study were also subjected to PCR analysis using SSR (using primers: EH-05, EH-02 and EH-07, see Table 2 for more details) to determine whether the DNA was degraded as shown in Figure 2. While no degradation was observed for DNA stored under different conditions, special in dry conditions ($\leq 5\%$ humidity). To assess its effects on DNA stability during storage at different conditions under controlled humidity. It was very interesting to note that DNA samples stored in the presence or absent of protectant additives exhibited no detectable degradation, whereas in the absent of, DNA was degraded and appeared as a smear on an agarose gel (Figure 2).

From the results obtained, the best way to protect DNA during storage was treated with trehalose. These results agreed with Ivanova and Kuzmina (2013) and Clermont et al. (2014). The results presented demonstrate the properties of protective be not affected by storage extreme conditions but guaranteeing for long term without degradation of DNA as well as the stored DNA can be used in downstream applications such as PCR.

Trehalose is well known agent for cryopreservation and lyophilization of biological samples (Taylor et al., 1994; McGinnis et al., 2005), DNA (Smith and Morin, 2005; Zhu et al., 2007) and RNA (Jones et al., 2007). Among the most commonly used disaccharides (sucrose and trehalose), trehalose is preferable for stabi-

lization of biomolecules due to its higher transition temperature. It stabilizes DNA due to its ability to form tight hydrogen bonds to the phosphate groups, which leads to shielding of the large phosphate-phosphate repulsion. As well as, it probably interacts with other polar groups of DNA that makes trehalose a water-like solvent for DNA and stabilizes the base stacking during and after dehydration (Zhu et al., 2007). The advantages of trehalose can be summarized as follows: more flexible formation of hydrogen bonds with proteins and DNA due to the absence of internal hydrogen bonds; less hygroscopicity; low chemical reactivity; and prevention of water plasticizing the amorphous phase partly by forming trehalose-protein-water microcrystals (Crowe et al., 1992; librizzi et al., 1999).

PVA is a polymer which has many desirable characteristics specifically for various applications (Hassan et al., 2000). It can be successfully used for PCR typing analysis (Schyma et al., 1999). The studies of PVA-DNA cryogel have demonstrated strong interaction between DNA and PVA (Papancea et al., 2008). The hydrogen bonding between DNA and PVA enables preparation of PVA-DNA nanoparticles suitable for gene and protein delivery applications (Liao et al., 2005). Besides, this compound is not toxic, does not affect enzymatic reactions, and keeps some amount of water molecules tightly attached even in dehydrative solvent which is advantageous for enzyme stabilization (Szczechsna-Antczak et al., 2002).

Figure (2) The banding profile detected by SSR primer for ten samples stored in different buffers at -20°C , -80°C and RT.

References:

- Adams, R.P. (1997). Conservation of DNA: DNA Banking. J. A. Callow, B. V. Ford-Lloyd and H. J. Newbury Biotechnology and Plant Genetic Resources. CAB International.
- Adams, R.P., Baker, L.E. and Pandey, R.N. (2011). [Seventeen years storage of Juniper and Spinach leaves in Alcohols: effects on DNA](#). Phytologia. Vol. 93(3): 283-292.
- Adams, R.P., Do, N. and Ge-lin, C. (1992). [Preservation of DNA in plant specimens from tropical species by desiccation](#). Pp. 153-181 in [Conservation of Plant Genes, DNA Banking and In Vitro Biotechnology](#) (R. P. Adams and J. E. Adams, eds.). Academic Press Inc., San Diego, USA.
- Barracough, T.G. and Vogler, A.P. (2000). [Detecting the Geographical Pattern of Speciation from Species-Level Phylogenies](#). Am. Nat., (155): 419 – 434.
- Bittencourt, J.V.M. and Sebbenn, A.M. (2009). [Genetic effects of forest fragmentation in high-density Araucaria angustifolia populations in Southern Brazil](#). Tree Genetics & Genomes, (5): 573 – 582.
- Chapman M.A., Chang J., Weisman D., Kesseli R.V., Burke J.M. (2007). [Universal markers for comparative mapping and phylogenetic analysis in the Asteraceae \(Compositae\)](#). Theor. Appl. Genet. Vol. (115): 747 – 755.
- Clermont, D., Santoni, S., Saker, S., Gomard, M., Gardais, E. and Bizet, C. (2014). [Assessment of DNA Encapsulation, a New Room-Temperature DNA Storage Method](#). Biopreservation and Biobanking. Vol. 12 (3): 176- 183.
- Crowe, J.H., Hoekstra, F.A. and Crowe, L.M. (1992). Anhydrobiosis. Ann. Rev. Physiol. Vol. (54): 557–577.
- Doyle J.J., Doyle J.L., Brown A.H., Palmer R.G. (2002). [Genomes, multiple origins, and lineage recombination in the Glycine tomentella \(Leguminosae\) polyploid complex: histone H3-D gene sequences](#). Evolution, (56): 1388 – 1402.
- Efombagn I.B.M., Motamayor J.C., Sounigo O., Eskes A.B., Nyassé S., Cilas, Ch., Schnell, R., Manzanares-Dauleux, J.M. and Kolesnikova-Allen, M. (2008). [Genetic diversity and structure of farm and GenBank accessions of cacao \(Theobroma cacao L.\) in Cameroon revealed by microsatellite markers](#). Tree Genetics & Genomes. (4): 821 – 831.
- Hassan, C., Stewart, J. and Peppas, N. (2000). [Diffusional characteristics of freeze/thawed \(polyvinyl alcohol\) hydrogels: Applications to protein controlled release from multilaminar devices](#). European Journal of Pharmaceutics and Biopharmaceutics. Vol. (49): 161–165.
- Ivanova, N.V. and Kuzmina, M.L. (2013). [Protocols for dry DNA storage and shipment at room temperature](#). Molecular Ecology Resources, (13): 890 – 898.
- Joly S. and Bruneau A. (2004). [Evolution of triploidy in Apios americana \(Leguminosae\) revealed by genealogical analysis of the histone H3-D gene](#). Evolution, (58): 284 – 295.
- Jones, K.L., Drane, D. and Gowans, E.J. (2007). [Long-term storage of DNA-free RNA for use in vaccine studies](#). Bio. Techniques. Vol. (43): 675–681.
- Liao, Y-H., Brown, M.B., Jones, S.A., Nazir, T. and Martin, G.P. (2005). [The effects of polyvinyl alcohol on the in vitro stability and delivery of spray-dried protein particles from surfactant-free HFA 134a-based pressurized metered dose inhalers](#). International Journal of Pharmaceutics. Vol. (304): 29–39.
- Librizzi, F., Vitrano, E. and Cordone, L. (1999). [Dehydration and crystallization of trehalose and sucrose glasses containing carbonmonoxy-myoglobin](#). Biophysical Journal. Vol. (76): 2727–2734.
- Mattick, J.S., Ablett, E.M. and Edmonson, D.L. (1992). [The gene library - preservation and analysis of genetic diversity in Australasia](#). Pp. 15-35 in [Conservation of Plant Genes, DNA Banking and In Vitro Biotechnology](#) (R.P. Adams and J.E. Adams, eds.). Academic Press, San Diego, USA.
- McGinnis, L.K., Zhu, L.B., Lawitts, J.A., Bhowmick, S., Toner, M. and Biggers, J.D. (2005). [Mouse sperm desiccated and stored in trehalose medium without freezing](#). Biology of Reproduction. Vol. (73): 627 - 633.
- Moretzsohn M.C., Leoi L., Proite K., Guimarães P.M., Leal-Bertioli S.C., Gimenes, M.A., Martins, W.S., Valls, J.F., Grattapaglia, D. and Bertioli, D.J. (2005). [A microsatellite-based, gene-rich linkage map for the AA genome of Arachis \(Fabaceae\)](#). Theor. Appl. Genet (111): 1060 – 1071.
- Murray, M.G. and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res., (8): 4321 – 4325.
- National Research Council. (1993). [Managing Global Genetic Resources, Agricultural Crop Issues and Policies](#). National Academy Press, Washington D.C.
- Papancea, A., Valenta, A.J.M., Patachia, S., Miguel, M.G. and Lindman, B. (2008). [PVA-DNA cryogel membranes: characterization, swelling, and transport studies](#). Langmuir, (24): 273 - 279.
- Salamini, N., Hodkinson, T.R., Savolainen Coates, V. (2005). [Towards building the tree of life: a simulation study for all angiosperm genera](#). Syst. Biol. (54): 183-196.
- Schyma, C., Huckenbeck, W. and Bonte, W. (1999). [DNA-PCR analysis of bloodstains sampled by the polyvinyl-alcohol method](#). Journal of Forensic Science. Vol. (44): 95–99.
- Smith, S. and Morin, P.A. (2005). [Optimal Storage Conditions for Highly Dilute DNA Samples: A Role for Trehalose as a Preserving Agent](#). J. Forensic Sci. Vol. 50 (5): 1 – 8.
- Song Q.J., Marek L.F., Shoemaker R.C., Lark K.G., Concibido V.C., Delannay, X., Specht, J.E. and Cregan, P.B. (2004). [A new integrated genetic linkage map of the soybean](#). Theor. Appl. Genet., (109): 122-128.
- Szczęzna-Antczak, M., Antczak, T., Rzyńska, M. and Bielecki, S. (2002). [Catalytic properties of membrane-bound Mucor lipase immobilized in a hydrophilic carrier](#). Journal of Molecular Catalysis (B). Enzymatic. Vol. 19 (20): 261–268.
- Takayama, K., Tateishi, Y., Murata, J. and Kajita, T. (2008). [Gene flow and population subdivision in a pantropical plant with sea-drifted seeds Hibiscus tiliaceus and its allied species: evidence from microsatellite analyses](#). Mol. Ecol., (17): 2730 –

- 2742.
26. Taylor, D.J., Finston, T.L. and Hebert, P.D.N. (1994). [The 15% solution for preservation](#). Trends in Ecology and Evolution. Vol. (9): 230.
27. Whitlock, R., Hipperson, H., Mannarelli, M. and Burke, T. (2008). [A high-throughput protocol for extracting high-purity genomic DNA from plants and animals](#). Mol. Ecol. Resour. Vol. (8): 736-741.
28. Witono, J.R. and Kondo, K. (2006). [Modification of DNA isolation protocol from silica gel dried-leaf tissues of Pinanga \(Palmae\)](#). Berita. Biologi. Vol. (8): 91-97.
29. Xie, W.G., Zhang, X.Q., Cai, H.W., Liu, W. and Peng Y. (2010). [Genetic diversity analysis and transferability of cereal EST-SSR markers to orchardgrass \(Dactylis glomerata L.\)](#). Biochem. Syst. Ecol., (38): 740-749.
30. Zhu, B., Furuki, T., Okuda, T. and Sakurai, M. (2007). [Natural DNA mixed with trehalose persists in B-form double-stranding even in the dry state](#). J. Physic. Chem. Vol. (111): 5542–5544.